Long-Residence-Time Nano-scale Liposomal Iohexol for X-ray–based Blood Pool Imaging[1]

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Rationale and Objectives. Although soluble nonionic iodine compounds with low systemic toxic effects have been developed for use in computed tomography (CT), they have short residence times of a few minutes or mere seconds—insuf - cient time for blood pool imaging, even with high-speed multi–detector row spiral CT. Moreover, potential renal toxic effects preclude repeated administration of these contrast agents during imaging, as well as their use in patients with compromised renal function. The objective of this study was to develop and evaluate a CT contrast agent for blood pool imaging that remains in the blood for more than 3 hours and that is relatively nontoxic to the kidneys.

Materials and Methods. The authors assessed a liposomal iohexol formulation for its encapsulation ef ciency in terms of milligrams of iodine per milliliter of lipid formulation and for its stability in phosphate buffer solution and in human plasma in vitro. Using a rabbit model, they also assessed the formulation's in vivo stability, residence time, and enhancement of contrast on images of various organ systems.

Results. The formulation, which contained 34.8 mg of iodine per milliliter of liposomal iohexol solution, remained stable in blood plasma both in vitro and in vivo, after injection into rabbit vasculature. An intravenous dose of 475 mg of iodine per kilogram of body weight produced contrast enhancement in the rabbit model of approximately 130 HU in the aorta and liver cortex and approximately 100 HU in the kidney cortex. Contrast enhancement was maintained for 3 hours after injection, and minimal clearance of the contrast agent via the kidneys was observed.

Conclusion. The liposomal iohexol formulation tested in this study had a suf cient residence time for blood pool imaging in a rabbit model. Future experiments with long-residence-time iohexol formulations may lead eventually to applications in cardiac imaging and in early tumor detection.

Key Words. Computed tomography (CT), contrast media; contrast media, experimental studies.

Major technologic advances have been made in computed tomography (CT) with the introduction of spiral CT scanners and, subsequently, subsecond multi–detector row CT scanners (1); however, advances in contrast agents have been slow. The most recent major advance (de ned here as acceptance into routine clinical practice) in contrast agents occurred with the introduction of nonionic compounds that provide increased safety and a reduction in contrast material–induced alterations in physiology (2). The limitations of current x-ray contrast agents include rapid clearance (which in some applications may be an advantage), increasing viscosity with increased radiopacity, a tendency toward extravasation, toxic effects in pa-

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tients with compromised renal function, the possibility of allergic reactions, and a lack of speci city for differentiating tissues and measuring physiologic activity. If x-ray contrast agents can be formulated that avoid or overcome these limitations, then quantitative assessments of structure-function relationships would be achievable with the new generation of multi–detector row CT scanners. Such advances would further the ongoing revolution in CT.

Blood pool imaging has many applications, including early detection of tumors and evaluation of cardiac disease (3–5). Since tumor growth often coincides with angiogenesis (6), blood pool imaging can be used for detecting, characterizing, and assessing tumor aggressiveness and for monitoring the response to therapy. Blood pool imaging also can aid in the detection of structural and functional abnormalities such as those caused by thrombi or atherosclerotic lesions and can provide imaging guidance for minimally invasive treatment of cardiac disease.

While soluble nonionic iodine compounds with relatively low osmolarity have been developed for use in CT blood pool imaging (eg, iohexol, iopromide), they have very short residence times of a few heartbeats, and their clearance via the kidney can be accompanied by renal toxic effects (7,8). Blood pool imaging with these contrast agents, even with the use of high-speed multi–detector row CT scanners, is dif cult (9) because of their short residence time. In addition, the clearance of such contrast agents via the kidney precludes their use in patients with compromised renal function and in protocols involving repeated administration of contrast material.

For the past 20 years, liposomes have been considered promising carriers for various contrast agents designed for use in therapeutic, diagnostic, and preventive medicine (10,11); they are highly biocompatible, and they reduce systemic toxic effects by entrapping bioactive agents. Early investigations of the use of liposomes for CT contrast agent encapsulation reported the clearance of the liposomes within 1–2 hours (12–14). Liposomes have been fabricated that are about 200 nm in diameter, to allow for the encapsulation of high amounts of contrast agent (5,13). However, even when they have been sterically stabilized by the incorporation of polyethylene glycol (PEG) into their membranes (ie, PEGylated), these relatively large liposomes are rapidly recognized and sequestered by the cells of the reticuloendothelial system, which increases the rate of their clearance (15). Other attempts at fabricating long-residence contrast agents for use in CT have resulted in iodinated macromolecular formulations with half-lives of 30 minutes (16,17) and renal clearance.

In this study, we investigated the effects of encapsulation of the commonly used contrast agent iohexol in PEGylated liposomes with a diameter of approximately 100 nm for the achievement of longer in vivo residence times (11,12) and of clinically relevant levels of contrast enhancement with low renal clearance.

MATERIALS AND METHODS

Liposomal Iohexol Formulations

Liposomal iohexol was fabricated by methods similar to those described elsewhere (18). Brie y, a lipid mixture (200 mmol/L) consisting of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol (chol) and *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE-MPEG2000) in a 55:40:5 molar ratio was dissolved in ethanol at 65°C. The ethanol solution was then hydrated with iohexol (350 mg iodine per milliliter) for 1 1 2–2 hours. Liposomes were extruded with a 10-mL Lipex Thermoline extruder (Northern Lipids, Vancouver, British Columbia, Canada) with ve passes through a 0.2-μm Nuclepore membrane (Waterman, Newton, Mass) and seven passes through a 0.1-μm Nuclepore membrane (Waterman). Liposomes were then dialyzed overnight in a 300,000-molecular-weight-cutoff dialysis bag against phosphate buffer solution to remove the free iohexol. The size of the resultant iohexol-containing liposomes was determined by dynamic light scattering with the use of a modi ed BI-90 goniometer, 532-nm laser (JDS Uniphase; San Jose, Calif), Hamamatsu photomultiplier (supplied by Brookhaven, Long Island, NY), and Dynamic-Light-Scattering software, version 3.16 (Brookhaven). The osmolarity of the liposomal iohexol formulation was measured with a vapor pressure osmometer (Vapro; Wescor, Logan, Utah).

Encapsulation Efficiency and Stability in Vitro

The iohexol concentrations of the resultant liposomal formulations were determined by measuring the absorption of ultraviolet (UV) light at 245 nm with a UV-visible spectrophotometer. The in vitro stability of liposomal iohexol formulations was determined by measuring the leakage of iohexol from the liposomes both in phosphate buffer solution at 4°C and in plasma at 37°C. In a typical procedure, 1 mL of liposomal iohexol was placed in the previously described dialysis bag and dialyzed against 250 mL of phosphate buffer solution at 4°C. At each time

point (0, 1, 2, 3, 8, and 24 hours, and 3, 4, 5, 6, 8, 10, and 18 days), 1 mL of the dialysate was removed for UV absorption–based iohexol measurement. At least three measurements were obtained at each time point. After measurement, samples were returned to the phosphate buffer solution to maintain constant volume.

To measure stability in blood plasma, the liposomal iohexol formulations were dialyzed against 250 mL of phosphate buffer solution at 25°C for 1 hour to remove free iohexol. In these experiments, 1 mL of liposomal iohexol was placed in the previously described dialysis bag with 4 mL of human plasma and dialyzed against 250 mL of phosphate buffer solution at 37°C (a 1:4 ratio was chosen because it is close to the ratio of liposomal iohexol to total blood volume needed to achieve 200-HU enhancement in the blood pool). One milliliter of the external phase was removed at 0, 1, 2, 3, 4, 5, 6, and 8 hours and analyzed for UV absorption. Because plasma components (which have a nite absorbance of light at 245 nm) leaked from the dialysis bag, a control experiment also was performed in which a phosphate buffer–blood plasma mixture was dialyzed against phosphate buffer solution. The absorbance of the external phase was subtracted from that for the liposomal iohexol formulation, and the resulting absorbance traces were representative of the leakage of iohexol from liposomal iohexol formulations.

In Vivo Studies: Injection and Imaging of Liposomal Iohexol in a Rabbit

The University of Iowa Animal Care and Use Committee approved all procedures used in this study. One female rabbit weighing 2.2 kg was anesthetized with 35 mg/kg ketamine and 5 mg/kg xylazine given by intramuscular injection, followed by 2% iso urane vapor given by inhalation. After tracheal intubation of the rabbit and placement of a venous catheter in an ear vein, 20 mg pentobarbital was given intravenously. The animal's lungs were ventilated with a pressure-controlled ventilator set at a peak airway pressure of 15 cm H_2O and 25 breaths per minute. The rabbit was then transported to the CT scanner and given 0.25 mg of pancuronium (a muscle relaxant) to ensure minimal motion during image acquisition. Supplemental pentobarbital (10–20 mg) was given every 30–60 minutes.

An initial image volume of the chest and abdomen was obtained by using a four-section multi–detector row CT scanner (MX8000; Philips Medical Systems, Cleveland, Ohio) in spiral scanning mode, with the following parameters: 100 mAs, 120 keV, single-section equivalent pitch of 1.25, and section collimation and thickness of 1.3 mm. Images were reconstructed to t a 512 × 512 matrix within a 12-cm eld of view by using a standard reconstruction kernel (the "B" kernel). A 0.5-second gantry rotation speed was used. During each scanning sequence, the rabbit was held apneic for approximately 15 seconds (to accommodate scanning time), with airway pressure xed at 20 cm H_2O (ie, near total lung capacity) and with an underwater bubbler tube on the exhalation port. The scanning sequence was repeated twice, and each time it was preceded by manual injection of 15 mL of liposomal iohexol (34.8 mg iodine per milliliter) via the venous catheter. A total dose of 475 mg iodine per kilogram of body weight was given in each of the two injections. The scanning sequence was repeated approximately 12, 60, 90, 120, 150, and 180 minutes after the second injection of contrast material. After the last scanning sequence was completed (approximately $3\frac{1}{2}$ hours after the last injection of contrast agent), the animal was euthanized with an overdose of pentobarbital, and a nal scan was obtained with no motion artifacts and with the same airway pressure and image acquisition settings. Finally, a high-resolution scan was obtained by using an ultrasharp reconstruction kernel (a "D" kernel) and an image matrix of 1,024 × 1,024 to enable visualization of anatomic detail without the presence of cardiogenic motion. Because of the high heart rate in rabbits, cardiac gating during scanning of the live rabbit was not practical.

Image Reconstruction

Subsequent off-line reconstructions were performed for each of the scans by using the smallest eld of view (5 × 5 cm, 0.1-mm voxel size) for three-dimensional viewing of the heart. The enhanced heart chambers were visualized by selecting appropriate settings of the volume-rendering software on the Philips MXV workstation (version 4.1). Once the settings were established, the same volume-rendering and display settings were used for all time points. Additional structures were segmented at various time points.

Quantitative analysis was performed by locating regions of interest in the aorta, heart, kidney (medulla and cortex), liver, muscle, and spleen. Mean attenuation (in Houns eld units) was determined at each time point to enable tracking of any decay in contrast material concentration with time in each of these structures. Section number and location of the regions of interest within a section

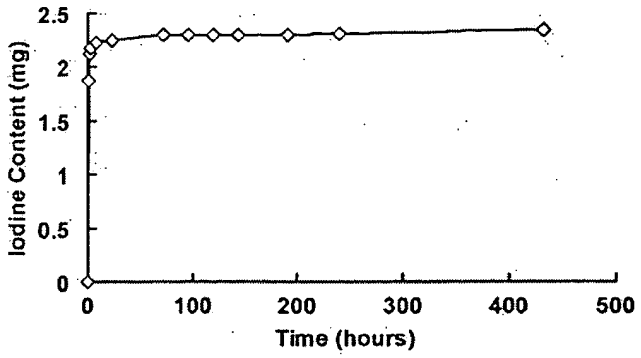


Figure 1. Graph of in vitro stability of liposomal iohexol dialyzed with phosphate buffer solution at 4°C. Total iodine content is 30 mg. ◇ = milligrams of iodine released.

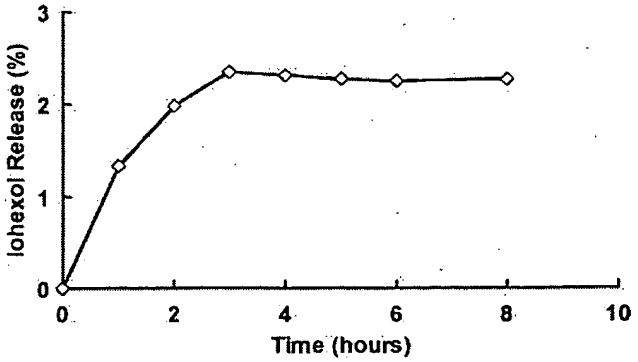


Figure 2. Graph of in vitro blood plasma stability of liposomal iohexol dialyzed with phosphate buffer solution at 37°C. Total iodine content is 28 mg. ◇ = milligrams of iodine released.

were adjusted for minor variations in anatomic con guration of the rabbit from one measurement time point to another.

RESULTS

Liposomal Iohexol Formulation

Liposomal iohexol formulations (DPPC/cholesterol/DSPE-MPEG2000 55:40:5) were prepared by high-pressure extrusion, and free iohexol was removed by overnight dialysis against the phosphate buffer solution. The average diameter of the liposomal iohexol capsules was 100.6 nm (standard deviation = 3.0 nm), as determined with dynamic light scattering. Different lipid hydration times (1^1_2 and 2 hours) resulted in different iohexol loading concentrations (30 and 34.8 mg iodine per milliliter, respectively). The 30-mg-iodine-per-milliliter liposomal iohexol formulation was used in the in vitro stability tests, and the 34.8-mg-iodine-per-milliliter liposomal iohexol formulation was used in CT scanning. The osmolality of the iohexol formulation was 305–315 mOsm/kg water.

In Vitro Stability of Liposomal Iohexol Formulation

The liposomal iohexol formulation was stable in phosphate buffer solution and in human blood plasma. The leakage curves of iohexol from liposomes in liposomal iohexol and in the iohexol-plasma mixture are shown in Figure 1 and Figure 2, respectively. As shown by the curve in Figure 1, stabilization of leakage occurred after 1 hour of dialysis of the liposomal iohexol formulation (30 mg iodine per milliliter) against 250 mL of phosphate buffer solution at 4°C. Leakage of only 7.4% of the total encapsulated iohexol was observed after 8 hours of equilibrium dialysis at 4°C, and leakage of only 7.8% was observed after 18 days. The shelf life of the liposomal iohexol formulation used in this study is therefore expected to be longer than 18 days.

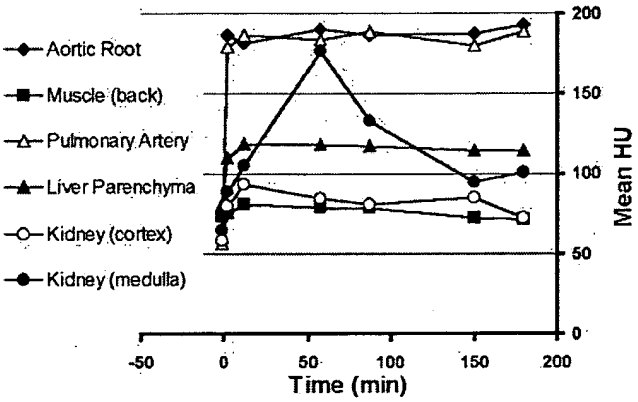


Figure 3. Time-attenuation curves of various regions of interest at different intervals after intravenous administration of liposomal iohexol in a 2.2-kg rabbit at a dose of 475 mg iodine per kilogram of body weight given in two incremental injections.

Figure 2 illustrates the stability of this liposomal iohexol formulation in human plasma. Liposomal iohexol that previously had been dialyzed against phosphate buffer solution for 1 hour was used in this experiment to determine the contribution of plasma to leakage of iohexol from the liposomes. The dialysis curve becomes stable after 3 hours, and the liposomal iohexol formulation exhibited a leakage of 2.3% of the total encapsulated iohexol after 8 hours.

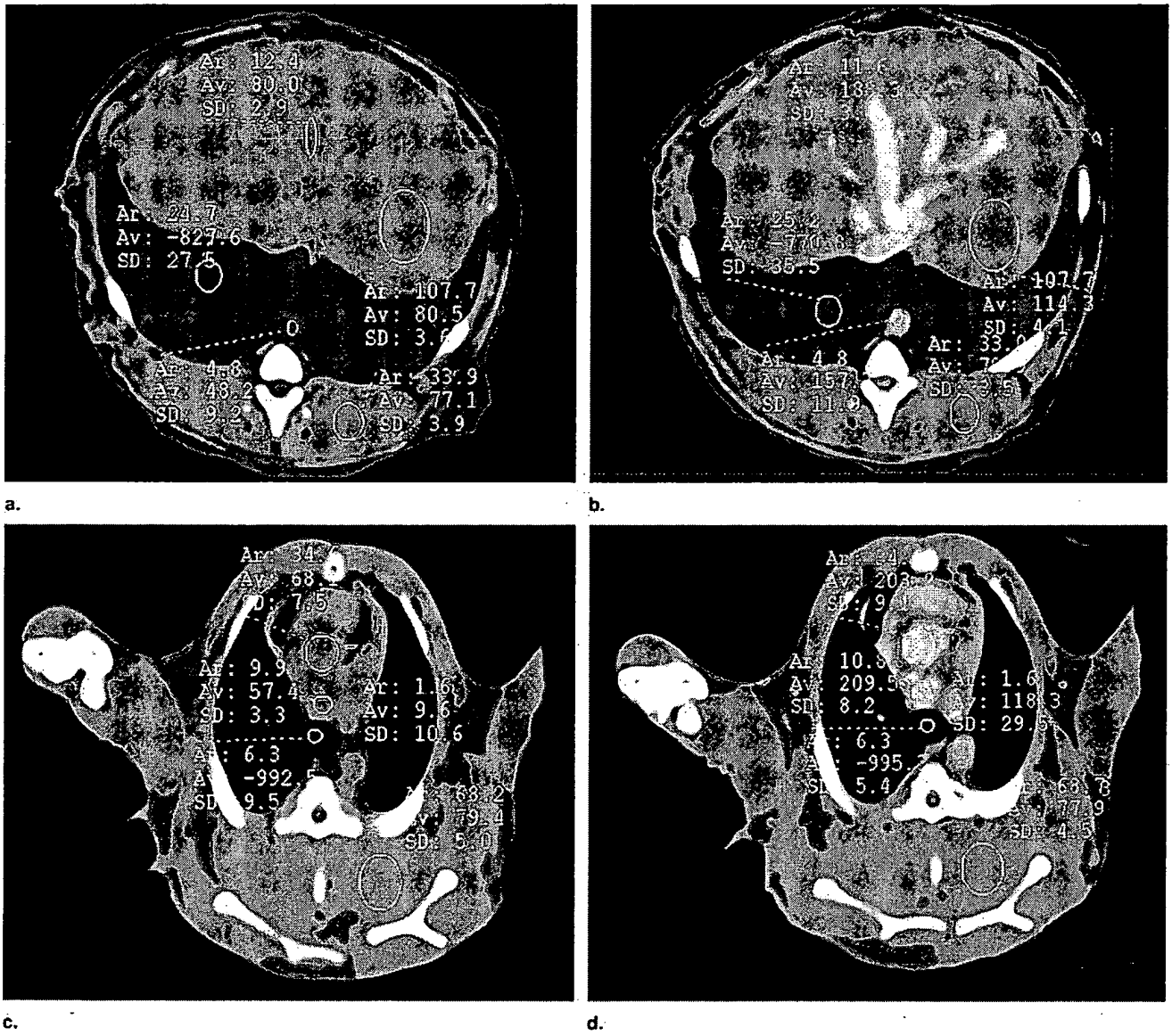


Figure 4. CT scans obtained in a 2.2-kg rabbit **(a, c)** before and **(b, d)** 2 hours 18 minutes after intravenous injection of liposomal iohexol containing 34.8 mg iodine per milliliter. Scans in **a** and **b** were obtained at the level of the liver; scans in **c** and **d** were obtained at the middle heart level.

CT Imaging

Image analysis was performed at regions of interest in the aorta, kidney (medulla and cortex), liver parenchyma, back muscle, left main coronary artery, pulmonary artery, and (as a control value) main stem bronchus. Mean attenuations were measured as Houns eld units at each time point to quantify the decay in contrast enhancement over time in each of these regions of interest. Figure 3 shows attenuation over time in various regions of interest. The average attenuation in the aorta, pulmonary artery, and liver cortex 3 1 2 hours after injection of contrast material was 200 HU (enhancement, 130 HU), and the attenuation in the kidney cortex was 75 HU (enhancement, 25 HU). Attenuation in the blood pool increased rapidly after contrast material injection and remained virtually constant for the approximately 3 1 2-hour observation period. A slight increase in attenuation in the liver parenchyma was observed. A transient increase in the kidney medulla was observed, indicating early clearance but little to no clearance later in the study. The small region of interest placed over the left main coronary artery indicated attenuation of 9 HU at baseline and peaked at a value of 118

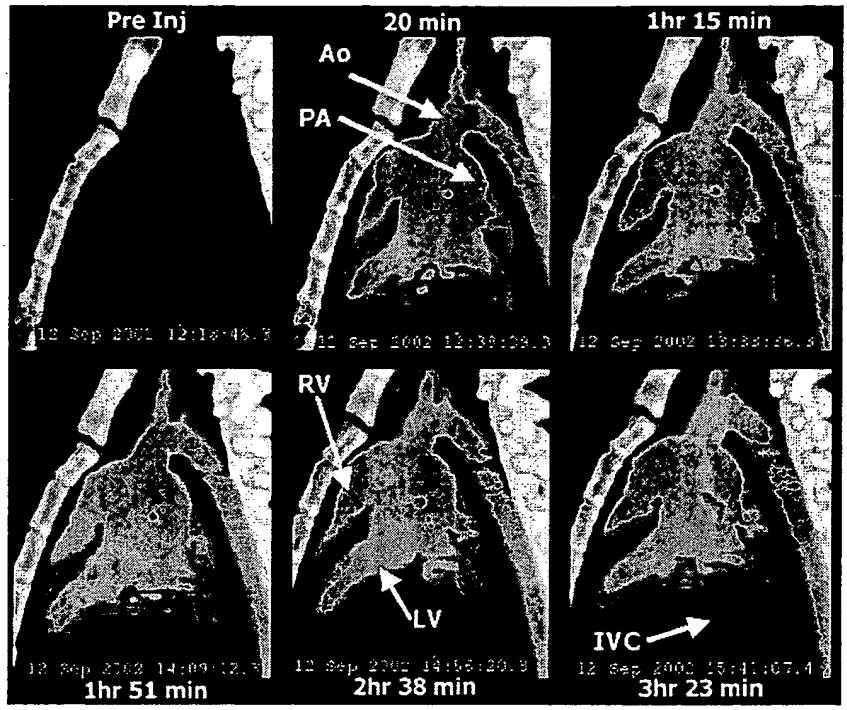


Figure 5. Volume-rendered CT images of rabbit heart obtained in vivo before (*Pre Inj*) and at various intervals after injection of liposomal iohexol. All volume-rendering parameters and display parameters were held constant for all time points. Note the absence of blood pool in the upper left panel and the persistent enhanced opacity of the blood pool up to the final panel at the lower right, which shows contrast enhancement at 3 hours 23 minutes after injection. Visible structures include the aorta (*Ao*), inferior vena cava (*IVC*), left ventricle (*LV*), pulmonary artery (*PA*), and right ventricle (*RV*).

HU. While the enhancement of contrast in the cardiac artery is consistent with that elsewhere in the blood pool, the absolute values are smaller than those for the rest of the blood pool, possibly because of partial volume effects resulting from surrounding structures. Figure 4 shows baseline and peak enhancement in the liver and the mid-heart levels.

Figure 5 shows volume images of the rabbit heart acquired before and at various intervals after administration of the liposomal iohexol. All display and rendering parameters are identical for all images. Because of the high heart rate in rabbits, however, cardiac gating was not performed during imaging; because of the relatively small amount of motion throughout the cardiac cycle, one can visualize the contrast-enhanced blood pool clearly. The anatomy of all four heart chambers and the associated great vessels is distinctly visible. These high-quality images demonstrate sustained contrast enhancement even 3 hours after the administration of the liposomal iohexol.

Figure 6 shows a thick-slab rendering of the heart obtained at high resolution after the rabbit was euthanized to eliminate cardiac motion. Figure 7 shows the left coronary artery of the rabbit under high magni cation 3 hours after the second injection of liposomal iohexol. The coronary artery, imaged in vivo during apnea, can easily be identi ed and showed an enhancement of 109 HU.

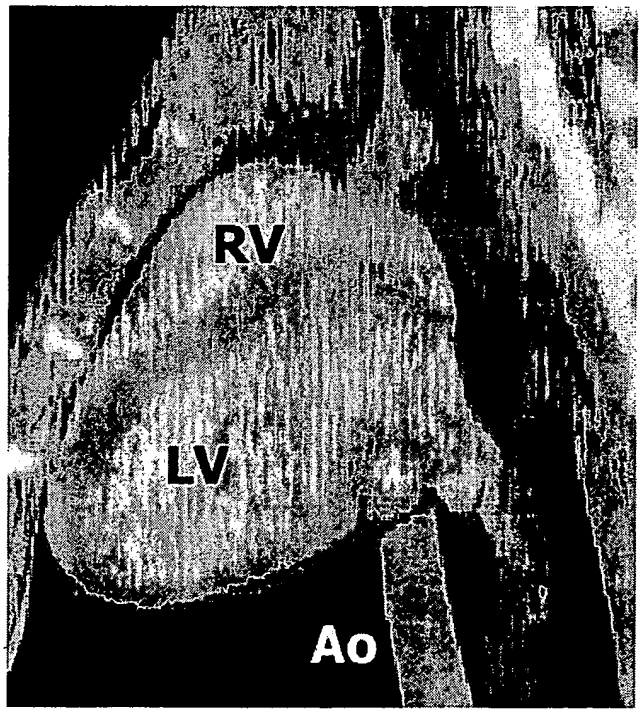
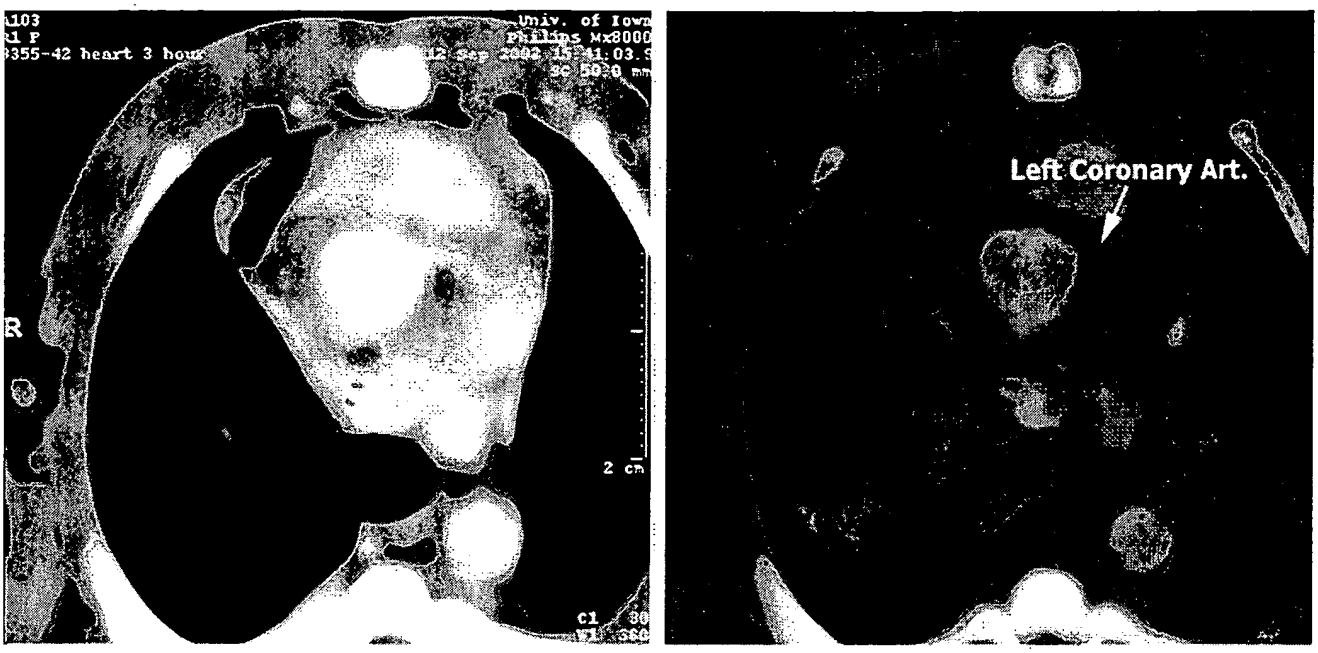


Figure 6. Thick-slab rendering of a high-resolution CT scan (24 line pairs per centimeter) obtained after death in the same rabbit as in Figure 5. The rabbit was sacrificed 3½ hours after the second injection of liposomal iohexol. *Ao* = aorta, *LV* = left ventricle, *RV* = right ventricle. Images were reconstructed to fit a 1,024 × 1,024 matrix with a 0.5-cm field of view.



a. b.

Figure 7. **(a)** CT section, 1.3 mm thick, obtained in vivo in the same rabbit as in Figure 5, 3 hours 18 minutes after the second injection of liposomal iohexol. Contrast enhancement in the left coronary artery is 109 HU. **(b)** Volume-rendered view of same image data set.

DISCUSSION

The goal of this study was to safely increase the residence time of a nonionic iodine-based contrast agent in the blood pool. To achieve long residence times, the liposomal iohexol formulation was modi ed through the incorporation of PEG into the liposomal membrane, creating sterically stabilized liposomes. The presence of PEG outer chains not only stabilizes the liposomal iohexol formulation but also helps avoid sequestration of the liposomes by the reticuloendothelial system.

In addition, many studies have demonstrated that drug delivery carriers that are smaller than 200 nm are taken up relatively slowly by the reticuloendothelial system, which may result in a longer residence time (19). The average diameter of the liposomes used in this study was 100 nm. We were able to use liposomes smaller than 200 nm successfully because we achieved loading ef ciencies of iohexol (30–35 mg iodine per milliliter) that were superior to those reported previously (15,19). However, our liposomal iohexol formulation necessitated an injection of a relatively large volume of the contrast material—15 mL in a 2.2-kg rabbit—nearly 14% of the blood plasma volume. The use of a contrast material volume of this magnitude clearly would not be acceptable in clinical practice; however, the volume could be reduced if the liposome concentration was increased or the amount of iodine entrapped per liposome was increased. Such preparations are the subject of ongoing work in our laboratories.

The results of in vitro stability tests indicate that the liposomal iohexol formulation remains stable at 4°C in phosphate buffer solution for at least 18 days. Thus, our current formulation is acceptable for short-term storage and research purposes, but it may need to be improved further to enable long-term storage (months). The results of in vitro stability testing show an additional 2.3% leakage of iohexol from the liposomes in blood plasma, beyond the 7.4% leakage observed during storage in phosphate buffer solution. These data suggest that our liposomal iohexol formulation would be about 90% encapsulated if it were stored for 18 days and then injected. This assumption is consistent with the approximately 10% free iohexol measured in our rabbit experiment (contributing to the renal clearance depicted in Fig 3).

There are two possible reasons for the leakage: (*a*) the difference in osmotic pressure between blood and the liposomal iohexol formulation or (*b*) shipping conditions during overnight transport of the liposomal iohexol formulation from Cleveland, Ohio, to Iowa City, Iowa. However, our measurements of leakage in vitro and in vivo

indicate that the renal "dose" incurred to achieve clinically relevant contrast enhancement in the blood pool would be substantially lower with our liposomal iohexol formulation than with free iohexol. To further reduce renal dose due to leakage, we are currently exploring other formulations to improve the lipid composition and thereby achieve greater stability of the contrast agent in blood plasma.

In our in vivo experiment, contrast enhancement of the aorta and pulmonary artery was used as an indicator of iodine concentration in the blood, because these regions of interest are blood lled and free of partial volume effects. The data presented in Figure 3 demonstrate the long residence time of our PEGylated formulation, which provided high contrast enhancement (Δ = 130 HU) for more than 3 hours. These results compare favorably with those reported by Sachse et al (5) and by Leike et al (13) on the basis of experiments with formulations combining iopromide with larger liposomes. In addition, the contrast enhancement in the muscle is low (Fig 3), suggesting that the liposomal iohexol is retained in the blood vessels and does not extravasate over the course of the 3 hours—perhaps partly because muscle is not highly vascular.

The contrast enhancement in the liver parenchyma suggests clearance of liposomal iohexol via the liver. The early enhancement observed in the kidney medulla is most likely from iohexol that leaked out of the liposomal encapsulation prior to injection (due to storage and transportation as described earlier) but also might be due to a slight instability of our formulation in blood plasma. When the values are calculated in relation to the total volume of the kidney, the transient increase in enhancement of the kidney medulla corresponds to about 10% of the total iohexol injected—a proportion that is consistent with the leakage of iohexol from the liposomes during storage and in blood plasma (approximately 10%) observed in our in vitro studies. However, it is important to note that even with this amount of leakage the renal dose incurred to achieve enhancement of approximately 100 HU is substantially lower than that incurred to achieve the same level of contrast enhancement with free iohexol. The clearance routes of PEGylated liposomes have been investigated thoroughly and are well established, and our formulation is very similar to that of the commercial products Doxil and Caelyx (sterically stabilized liposomal doxorubicin), as well as to that used in clinical studies of liposomal cisplatin (20). Our experience with the formulation used in this study suggests a substantial rate of hepatic clearance with extremely low renal clearance (20). Further investigation of actual reductions in renal toxic effects with the use of our formulation is needed.

These results demonstrate that a liposomal iohexol formulation can maintain a required level of contrast agent in the blood and organ for extended time intervals, resulting in better blood pool images. Compared with results reported previously for another liposomal formulation (5,13), our results show a far higher blood pool residence time, primarily due to two factors—a more favorable composition (ie, PEGylation) and smaller size (100 nm) of lipsosomes in our formulation. Sustained enhancement of blood pool contrast holds great promise for many applications, including cardiovascular, oncology, and neurology-based applications. Additionally, the demonstrated liposomal formulation can be coupled with antibody labeling methods to target contrast enhancement. We believe that this study demonstrates that, coupled with the increased spatial and temporal resolution offered by advances in multi–detector row CT, x-ray methods hold great promise for further advances in the realm of not only anatomic but also functional and molecular imaging applications.

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